Global transcriptome analysis of the heat shock response of *Bifidobacterium longum*

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Abstract

Bifidobacteria are natural inhabitants of the human gastrointestinal tract and have been widely used as functional foods in different products. During industrial processing, bacterial cells undergo several stresses that can limit large-scale production and stability of the final product. To better understand the stress-response mechanisms of bifidobacteria, microarrays were used to obtain a global transcriptome profile of *Bifidobacterium longum* NCC2705 exposed to a heat shock treatment at 50 °C for 3, 7 and 12 min. Gene expression data highlighted a profound modification of gene expression, with 46% of the genes being altered. This analysis revealed a slow-down of *Bi. longum* general metabolic activity during stress with a simultaneous activation of the classical heat shock stimulon. Moreover, the expression of several genes with unknown function was highly induced under stress conditions. Three of these were conserved in other bacteria species where they were also previously shown to be induced by high temperature, suggesting their widespread role in the heat stress response. Finally, the implication of the *trans*-translation machinery in the response of *Bi. longum* cells to heat shock was suggested by the induction of the gene encoding the tmRNA-associated small protein B (SmpB) with concomitant high constitutive expression of the tmRNA gene.

Introduction

Bifidobacteria are Gram-positive, heterofermentative and anaerobic bacteria that are found as natural inhabitants in the gut of humans and other warm-blooded animals (Ventura *et al.*, 2004b). The interest in bifidobacteria has grown significantly mainly because their presence has been associated with a healthy microbiota, which led to their widespread use as functional food supplements (Picard *et al.*, 2005; Chermesh & Eliakim, 2006; Szajewska *et al.*, 2006).

To survive in the harsh gastrointestinal tract conditions, these microorganisms need to be able to adapt to a competitive and changing environment. Furthermore, for industrial applications, selected strains must respond to the adverse conditions encountered during the production process, for instance high temperatures during spray drying (Simpson *et al.*, 2005) as well as during storage of the final product. The importance of inducible cell-protective systems for survival under stressful conditions has been shown in bifidobacteria (Schmidt & Zink, 2000; Maus & Ingham, 2003; Saarela *et al.*, 2004) and in other microorganisms (Flahaut *et al.*, 1996; Saarela *et al.*, 2004). Moreover, the mechanisms underlying these stress responses have been extensively investigated in many bacterial species (Richmond *et al.*, 1999; Yura *et al.*, 2000; Helmann *et al.*, 2001; Stewart *et al.*, 2002; Weiner *et al.*, 2003; De Angelis & Gobbetti, 2004; Gao *et al.*, 2004; Barreiro *et al.*, 2005). However, relatively little is known for bifidobacteria, partly due to the paucity of efficient molecular genetic tools for the analysis of gene functions. The analysis of the genome sequence of *Bifidobacterium longum* NCC2705 (Schell *et al.*, 2002) revealed a number of physiological traits that partially explain its successful adaptation to the gut environment and the identification of conserved bacterial stress response genes allowed predicting some of its stress-protective mechanisms (Klijn *et al.*, 2005). Using a proteomic approach, several stress-induced proteins have been identified in *Bi. longum* cells upon exposure to bile salts (Sanchez *et al.*, 2005; Savijoki *et al.*, 2005) or high temperatures (Savijoki *et al.*, 2005). These included general stress-related

chaperones, proteins involved in transcription and translation and few proteins from different metabolic pathways. A recent proteomic analysis performed on *Bi. longum* cells grown on different culture media showed that 21% of the predicted CDS were expressed and few of them were shown to display substrate-dependent expression (Yuan *et al.*, 2006). Finally, a series of studies have been recently published describing the genetic organization and transcriptional regulation of few major stress-related chaperones of *Bifidobacterium breve* (Ventura *et al.*, 2004a, 2005a–e; Klijn *et al.*, 2005). However, till date there have been no reports on genome-wide gene expression changes induced in a *Bifidobacterium* strain upon exposure to a stress stimulus.

In this study, whole-genome DNA microarrays covering 97% of all *Bi. longum* NCC2705 CDS were used to analyse the time-dependent gene-expression changes induced in response to a severe sublethal heat shock. This allowed us to identify the *Bi. longum* heat stress response mechanisms and to compare them with other bacterial species.

Materials and methods

Bacterial growth conditions, heat stress and cell counting

Bifidobacterium longum NCC2705 was inoculated at 1% from an overnight culture into a 7 L bioreactor (new MBR Bioreactor AG, Zurich, Switzerland) filled with 4 L of MRS medium (BD, Basel, Switzerland). Cultures were grown at 37 °C under anaerobic conditions. Bacterial cells suspensions (400 mL) were harvested in mid-exponential phase ($OD_{600\,nm}$ of 0.7–0.8) and split into two aliquots that were immediately centrifuged at room temperature (RT) for 5 min at 3600 ***g***. The supernatants were rapidly removed and the pellets suspended in prewarmed MRS medium at either 37 or 50 °C and incubated in a water bath at the corresponding temperature. Ten milliliter culture aliquots were collected simultaneously at 3, 7, 12 and 19 min from the two cell suspensions and immediately centrifuged at RT for 5 min at 3600 ***g***. Bacterial pellets were then immediately frozen in liquid nitrogen. For estimation of cell survival, culture aliquots were collected from the different time points and plated at three dilutions on MRS agar and incubated for 2 days in anaerobic jars before cell counting.

Production of microarrays, RNA extraction and hybridization

Glass microarrays spotted with 200–800 bp-long PCR-based amplicons targeting *c.* 97% of all *Bi. longum* NCC2705 CDS were described elsewhere (Parche *et al.*, 2006). RNA extraction, RNA quality checks, labeling and hybridization were performed as previously described (Parche *et al.*, 2006). Hybridizations were performed using RNAs from three independent experimental biological replicates for each time point and technical hybridization replicates were performed for some of the RNAs. Scanarray 4000 (Packard Biochip Technologies, Billerica, MA) was used for scanning of microarrays.

Microarray data analysis

Imagene 5.6 (Biodiscovery, El Segundo, CA) was used to extract data from the scanned images. The local average background was subtracted from the average spot signal values. Spots with low signal intensities for both Cy3 and Cy5 (i.e. less than threefold the average local background) were excluded, whereas spots displaying low signal intensities in only one channel were retained if the signal in the other channel was at least fivefold higher than the corresponding average local background signal. A within-array normalization was performed on the $\log_2$-transformed gene expression ratios (*M*-values) to bring the median of combined *M*-values within each array to zero. *M*-values from spot duplicates on each array were averaged first and then *M*-values from independent technical hybridization replicates were combined. As a result of these data-processing steps, *M*-values from the three biological replicates were obtained for the majority of the genes for each time point. Statistical analysis was based on an empirical Bayes method (Lönnstedt & Speed, 2002; Hatfield *et al.*, 2003; Smyth, 2004) implemented in the software package LIMMA for the R-computing environment (www.r-project.org). The threshold of significance was set to $1/no$ ($\alpha = \sim 0.0007$), where *no* is the number of genes with an expression value in at least two out of three biological repeats. Finally, genes were considered as differentially expressed if their absolute $\log_2$-transformed signal ratios were greater than 1 and their *P*-values smaller than α.

Quantitative reverse transcriptase-PCR (qRT-PCR)

Oligonucleotide primers (Table S1) were designed with the PRIMER EXPRESS software (Applied Biosystems, Foster City, CA). Validation of microarray data was carried out by qRT-PCR measurements on 11 genes selected according to their microarray gene expression values including four constitutively expressed (BL0118, BL0274, BL0301, BL1800), five significantly induced (BL0517, BL0519, BL1180, BL1250, BL1672) and two significantly repressed genes (BL0279, BL1182). cDNA was synthetized according to the protocol recommended by the supplier using random hexamers and the TaqMan Reverse Transcription Reagents (Applied Biosystems). Real-time PCR was performed on an ABI PRISM 7000 machine (Applied Biosystems) using the SYBR Green

PCR Master Mix (Applied Biosystems), following the recommended protocol. Quantitative values were obtained using the comparative threshold-cycle method (ΔC_T). Gene expression was assayed in RNAs from control and heat shock-treated samples from all three experimental replicates. Quantification of relative expression of *ssrA* and *smpB* (BL1180) genes was performed using the $\Delta\Delta C_T$ normalization method using three housekeeping genes (BL0118, BL0274, BL0301) that were previously selected based on microarray data showing their constitutive expression under different experimental conditions (data not shown).

Operon prediction and classification of *Bi. longum* genes into functional categories

The number of *Bi. longum* genes organized in operons was identified using the method recently published by Price *et al.* (2005). Classification of the NCC2705 genes into different clusters of orthologous groups (COGs) was based on the most recent COG classification system (66 genomes; http://www.ncbi.nlm.nih.gov/COG). Percentages of differentially expressed genes in each COG functional category were calculated by dividing the number of significantly induced/repressed genes in each category by the total number of retained genes in the corresponding category (genes were retained if expression data from at least two experimental replicates were available). Statistical analysis of the differential expression observed in each COG category was based on hyper-geometric distribution. The *P*-values assigned to the different COG categories express the significance of the difference observed between the number of differentially expressed genes measured in a given COG category and the overall level of differential expression.

Results and discussion

Survival of *Bi. longum* following heat shock

To study the changes in gene expression occurring in *Bi. longum* in response to heat stress, optimal sublethal stress conditions were experimentally defined in order to induce extensive differential gene expression without compromising the viability of the cells. After preliminary tests at different temperatures, it was decided to apply a temperature of 50 °C to bacterial suspensions harvested in mid-exponential phase. Samples were collected 3, 7, 12 and 19 min after beginning of the heat shock and cell survival was assessed by counting cfu. No significant difference in survival rates was observed between control and heat-treated cells during the first 12 min (Fig. S1). However, after 19 min a reduction of cell viability was observed in one of the three experimental replicates (Fig. S1; -0.75 log-units), and hence it was decided that the focus of transcriptome analysis would be on the first 12 min.

Bifidobacterium longum rapidly reacts to heat treatment with a pronounced change in gene expression

The complete list of the average gene expression values obtained at 3, 7 and 12 min is presented in Table S2. Average expression data were further considered only for the genes exhibiting significant expression in at least two biological replicates (hereinafter defined as the transcriptionally active genes). This condition was fulfilled by 83% of all *Bi. longum* NCC2705 genes. Microarray data were validated by qRT-PCR measurements on eleven genes revealing a high level of correlation ($R^2 = 0.93$) between the two methods (Fig. S2). Figure 1a summarizes the number of genes differentially expressed at each time point. In total, 442 genes (206 induced and 236 repressed) at 3 min, 655 genes (314 induced and 341 repressed) at 7 min and 625 genes (290 induced and 335 repressed) at 12 min displayed an absolute differential expression $\geq$ twofold in response to the applied heat stress. This corresponds to 56% of the transcriptionally active genes being differentially expressed at least at one time point, i.e. 46% of all *Bi. longum* genes. According to an automated operon prediction method (Price *et al.*, 2005), 52% of the differentially expressed genes were organized in 215 different operonic structures. It is noteworthy that the lowest inter-experimental variability was observed at 7 min, a time point that is sufficiently advanced to enable establishment of a viable and uniformly responding cell population (see distribution of statistically significant differentially expressed genes, Fig. 1b). The impact of heat treatment on *Bi. longum* gene expression is more pronounced compared with data obtained in other species. For instance, when *Ba. subtilis* (Helmann *et al.*, 2001), *Escherichia coli* (Richmond *et al.*, 1999) and *Campylobacter jejuni* (Stintzi, 2003) were subjected to heat treatments of 48, 50 and 42 °C, respectively, only up to 20% of the genes were found to be differentially expressed. Moreover, in contrast to previous studies showing a transient transcriptome reorganization upon exposure to high temperature (Helmann *et al.*, 2001; Stintzi, 2003; Gao *et al.*, 2004), in *Bi. longum* most of the genes which were differentially expressed, remained induced and repressed throughout the time course. The most likely explanation for these differences is that the experimental stress conditions of this study were closer to the lethal threshold. Furthermore, different mechanisms of transcriptional regulation between these phylogenetically distant bacterial species might also explain the observed major effect of a heat treatment on *Bi. longum* transcriptional profile. To obtain an overview of the impact of high temperature on bacterial physiology, NCC2705 differentially

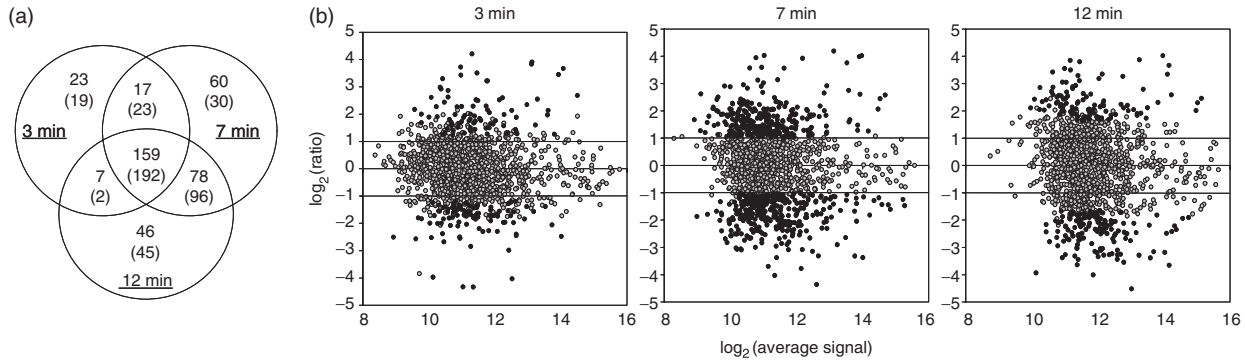

Fig. 1. Impact of the heat shock treatment on the global gene expression levels in *Bifidobacterium longum* after 3, 7 and 12 min at 50 °C. Only genes with expression data from at least two experimental replicates are shown. (a) Venn diagram displaying the number of differentially expressed genes at each time point; numbers in parentheses represent repressed genes ($\log_2$-transformed ratio ≤ -1), while the other numbers show induced ones ($\log_2$-transformed ratio ≥ 1). Genes that were found to be up or downregulated in two or three time points are indicated in the intersections. (b) 'M vs. A' plots displaying the global distribution of relative gene expression values [$\log_2$(ratio)] as a function of the $\log_2$-transformed average absolute gene expression levels in control and heat-treated cells. The horizontal lines indicate the threshold of twofold induction, respectively repression. Black dots indicate genes displaying statistically significant differential expression ($P < 0.0007$).

expressed genes were grouped into functional categories according to the COG classification system (Tatusov *et al.*, 1997). A clear bias towards heat-dependent regulation of genes involved in few specific cellular processes was observed (Fig. 2) and the functional categories exhibiting significantly different percentages of regulated genes will be discussed individually in the following sections.

Extensive downregulation of genes involved in cell growth

The majority of the genes belonging to COG categories J (translation, ribosomal structure and biogenesis) were repressed 7 min after exposure to high temperature conditions (Fig. 2). This category contains mainly genes encoding proteins involved in the translation machinery, such as ribosomal proteins and aminoacyl-tRNA synthetases and several of them are also found among the genes exhibiting the strongest level of transcriptional repression (Table S3). A similar observation was also reported for the food-borne pathogen *C. jejuni* (Stintzi, 2003). This down-regulation of ribosomal genes could represent a survival strategy of stressed bacteria that repress their most energy-demanding cellular processes for the benefit of other protective functions. In line with this hypothesis, genes involved in cell division and chromosome partitioning (COG category D) were also rapidly down-regulated. These observations probably reflect a heat-induced halt of *Bi. longum* cell division. In fact, when *Bi. longum* was inoculated on plates and incubated at different temperatures ranging from 37 to 50 °C, no growth was observed at temperatures equal to or higher than 45 °C (data not shown). Similarly, data from a previous study on another heat-stressed *Bi. longum* strain (Savijoki *et al.*, 2005) showed that shifting the cells from 37 to 47 °C resulted in a 50–80% reduction of the growth rate. Finally, genes encoding proteins involved in other essential processes linked to an active metabolic state like cell-envelope biogenesis (M), energy production and conversion (C), and nucleotide transport and metabolism (F) were also primarily repressed.

Induction of the *dnaK* and *groE* chaperone systems

A statistically significant overrepresentation of induced genes was furthermore found amongst members of the COG functional category O (posttranslational modification, protein turn-over, chaperones), which includes most genes belonging to the classical and extensively studied heat shock stimulon (Fig. 2). The *dnaK* operon (BL0516–BL0520), which includes genes encoding the major molecular chaperones DnaK, GrpE, DnaJ as well as the transcriptional repressor HspR, were among the most highly and rapidly induced genes. Similarly, the genes encoding the second major molecular chaperone system, namely *groEL* (BL0002) and *groES* (BL1558) and the corresponding transcriptional regulator HrcA (BL0718), were also induced but at lower levels (Tables 1 and 2). In *Bifidobacterium breve*, the *dnak* operon was not induced at mild heat shock temperatures of 43 °C (Ventura *et al.*, 2005e) while members of the *groE* machinery were shown to display the highest expression levels at 43 °C in a range from 37 to 50 °C (Ventura *et al.*, 2004a). The different regulatory patterns displayed by these two major chaperone systems probably reflect their distinct and complementary roles in different stress responses as already described in other bacterial species (Susin *et al.*, 2006). The role of *dnaK* in survival to heat stress was recently confirmed using a *Bi. longum* NCC2705 mutant

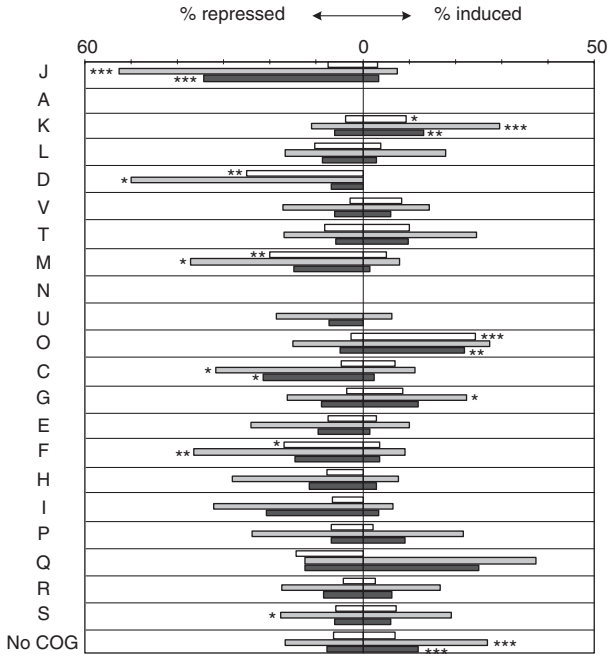

Fig. 2. Differentially expressed genes categorized by functional classification according to the COG annotation at 3 (white bars), 7 (grey bars) and 12 min (black bars) after beginning of the heat shock. Percentages of induced, respectively repressed, genes in each category was calculated by dividing the number of significantly induced/repressed genes in each category by the total number of retained genes in the corresponding category. *, statistical significance (*, *P*-value $\leq$ 0.05; **, *P*-value $\leq$ 0.005; ***, *P*-value $\leq$ 0.0005). Functional categories: J, translation, ribosomal structure and biogenesis; A, RNA processing and modification; K, transcription; L, replication, recombination and repair; D, cell cycle control, cell division, chromosome partitioning; V, defense mechanisms; T, signal transduction mechanisms; M, cell wall/membrane/envelope biogenesis; N, cell motility; U, intracellular trafficking, secretion, and vesicular transport; O, posttranslational modification, protein turnover, chaperones; C, energy production and conversion; G, carbohydrate transport and metabolism; E, amino acid transport and metabolism; F, nucleotide transport and metabolism; H, coenzyme transport and metabolism; I, lipid transport and metabolism; P, inorganic ion transport and metabolism; Q, secondary metabolites biosynthesis, transport and catabolism; R, general function prediction only; S, function unknown.

constitutively overexpressing the *dnaK* operon, which showed significantly improved resistance to high temperature (B. Berger, unpublished results).

Regulation of proteolytic enzymes

None of the genes encoding the subunits of the Clp protease complex were induced upon heat shock, namely the substrate-recognition subunits ClpC (BL0010) and ClpX (BL0943) and the genes encoding the proteolytic subunits ClpP1 (BL0944) and ClpP2 (BL0945) (Table 1). Similarly, *Bifidobacterium breve clpC*, *clpP1* and *clpP2* genes were recently shown to be induced in response to moderate heat shock regimens, but not at 50 °C (Ventura *et al.*, 2005b, d). The constitutive expression of these genes was unexpectedly accompanied by strong induction of the *Bi. longum* transcriptional activator ClgR (BL1414; Tables 1 and 2) that was proposed to be involved in the induction of the ClpP locus in *Bifidobacterium breve* under moderate temperature-shock conditions (Ventura *et al.*, 2005b, d). HtrA (BL0555), a widely distributed serine protease exhibiting both chaperone and protease activities in many bacteria (Pallen & Wren, 1997), was strongly induced at all time points in *Bi. longum* (Tables 1 and 2), suggesting its likely implication in the degradation of heat-induced misfolded proteins.

Induction of transcriptional regulators

As shown in Fig. 2, a significant overrepresentation of induced genes was found within the COG category K (transcription) including 30 induced transcriptional regulators, one probable serine/threonine-protein kinase (BL1198) and a transcriptional regulator belonging to the ROK family (BL0074) (Table S4). The list of induced transcriptional regulators included 2 two-component system (TCS)-response regulators (BL0316, BL0903), which displayed similar induction levels as the corresponding membrane-bound sensor histidine kinases (BL0317 and BL0902; see Table S2). TCS are well-known signal transduction systems employed by bacteria to detect environmental changes and mediate differential gene expression (Mascher *et al.*, 2006). Surprisingly, 10 LacI-type sugar-responsive-repressors were also found among the induced regulators suggesting an effect of the heat treatment on carbohydrate metabolism. Unfortunately, a precise functional role could be assigned only to few regulators including the above-mentioned HspR and HrcA (Narberhaus, 1999) and the transcriptional repressor LexA (BL1310) (Table 1). In *E. coli* and in several other bacterial species, LexA controls the SOS response system, including genes involved in inhibition of cell division, error-prone replication or excision repair (Fernandez De Henestrosa *et al.*, 2000; Campoy *et al.*, 2005). LexA was shown to interact with the RecA protein, which acts as a sensor of environmental stresses inducing DNA damage and promotes the autocatalytic cleavage of LexA, hence preventing its binding to target recognition sequences. In this study, *recA* (BL1415), *recX* (BL1416), another member of the RecA-mediated signaling pathway, and *recN* (BL1043), which encodes a protein involved in DNA repair were also slightly induced upon heat stress. Taken together, these results indicate that stressed *Bi. longum* respond to changes/defects/errors in their chromosomal DNA, by induction of their SOS response system.

Table 1. Overview of gene expression ratios of potential stress-related genes

Gene	Ratio† 3 min	7 min	12 min	Function
BL0001	2.4	3.6*	3.3*	Cold shock protein (CspA)
BL0002	5.8*	4.5*	4.3*	Chaperone protein (GroEL)
BL0007	1.0	1.5	1.5	Cold shock protein (CspB)
BL0009	0.9	0.9	0.8	Universal stress protein
BL0010	0.6	0.5	0.5	ATP-dependent endopeptidase clp ATP-binding subunit (ClpC)
BL0094	1.2	0.8	1.0	ATP-dependent endopeptidase
BL0516	14.2*	13.5*	16.2*	Heat-inducible transcriptional repressor (HspR)
BL0517	14.9*	18.2*	14.6*	Chaperone protein (DnaJ)
BL0519	10.9*	15.8*	12.7*	GrpE protein (HSP-70 cofactor)
BL0520	12.7*	16.2*	10.1*	Chaperone protein (DnaK)
BL0551	1.2	1.2	1.3	Possible endopeptidase (HtpX)
BL0555	6.4*	6.0*	5.5*	DO serine protease (DegP or HtrA)
BL0576	9.8*	15.4*	13.9*	Probable HSP20-family heat shock chaperone
BL0718	3.8*	3.6*	4.2*	Heat-inducible transcription repressor (HrcA)
BL0719	1.5	1.1	0.8	Chaperone protein (DnaJ)
BL0943	0.9	0.4	0.9	ATP-dependent endopeptidase clp ATP-binding subunit (ClpX)
BL0944	0.8	0.5	0.5	ATP-dependent endopeptidase clp proteolytic subunit (ClpP)
BL0945	0.8	0.6	0.6	ATP-dependent endopeptidase clp proteolytic subunit (ClpP)
BL1043	3.7*	3.7*	3.3*	DNA repair protein (RecN)
BL1180	4.4*	6.1*	6.0*	SsrA-binding protein (SmpB)
BL1250	18.4*	16.3*	11.2*	Endopeptidase Clp ATP-binding chain B (ClpB)
BL1310	3.9*	4.4*	3.3*	SOS-response transcriptional repressor (LexA)
BL1357	1.6	1.6	2.0	RNA polymerase ECF-type sigma factor
BL1358	3.5*	4.2*	3.1	ECF-type sigma factor negative effector
BL1414	9.3*	15.1*	9.6*	Transcriptional activator (ClgR; probe 1)
BL1414	2.9*	3.1*	2.8	Transcriptional activator (ClgR; probe 2)
BL1415	2.1	1.8	2.0	RecA protein
BL1416	3.0	2.3*	2.0	RecX protein
BL1418	3.0*	3.3*	2.4	Ribosome-associated factor Y
BL1428	1.2	1.2	1.2	RNA polymerase principal sigma factor; sigma 70
BL1439	0.9	0.9	1.2	GTP pyrophosphokinase (RelA)
BL1558	5.7*	4.1*	4.0*	10 kDa chaperonin (GroES)
BL1664	1.6	1.3	1.1	Widely conserved protein in universal stress protein family

*Significantly induced genes ($P < 0.0007$).

†Ratio of gene expression between heat-shock treated cells and control cells.

Induction of *trans*-translation protein salvage pathway

Ribosome 'stalling' can occur in bacteria for different reasons, e.g. as consequence of low tRNA levels, lack of stop codons in truncated mRNAs or inefficient translation termination. In a process known as *trans*-translation, a tmRNA molecule (encoded by the *ssrA* gene) acts as tRNA and mRNA to append a peptide tag to the nascent polypeptide and 'rescue' the ribosome (Withey & Friedman, 2003; Moore & Sauer, 2005). The tagged proteins are then rapidly degraded by specific proteases. In *Ba. subtilis*, tmRNA is induced under severe stress conditions and its presence is required for efficient growth (Muto *et al.*, 2000). SmpB is a dedicated tmRNA-binding protein that is essential for the *trans*-translation process (Karzai & Sauer, 2001). Microarray data obtained in this study revealed a significant and strong induction of *smpB* (BL1180) at all time points (Tables 1 and 2) that was further confirmed by qRT-PCR. Because the original *Bi. longum* microarray design did not include *ssrA* gene-specific probes, qRT-PCR was used to assess the relative *ssrA* gene expression levels in heat shock treated and control cells. Surprisingly and in contrast to what was observed in *Ba. subtilis, ssrA* was strongly and constitutively expressed in *Bi. longum*. To the best of the authors' knowledge, this is the first report that describes the regulation of the tmRNA/SmpB system in response to heat stress to occur by high constitutive expression of the *ssrA* gene with a concomitant induction of the *smpB* gene. These results suggest that in *Bi. longum*, induction of SmpB is required under stressful conditions in order to effectively protect tmRNA molecules against degradation by endogenous

Table 2. Fifty genes displaying the strongest level of induction after 7 min at 50 °C

Gene	Ratio† 5 min	7 min	12 min	Function
BL0517	14.9*	18.2*	14.6*	Chaperone protein (DnaJ)
BL1250	18.4*	16.3*	11.2*	Endopeptidase Clp ATP-binding chain B (ClpB)
BL0520	12.7*	16.2*	10.1*	Chaperone protein (DnaK)
BL0519	10.9*	15.8*	12.7*	GrpE protein (HSP-70 cofactor)
BL0576	9.8*	15.4*	13.9*	Probable HSP20-family heat shock chaperone
BL1414	9.3*	15.1*	9.6*	Possible DNA binding protein (transcriptional regulator)
BL0516	14.2*	13.5*	16.2*	Heat shock transcriptional regulator (HspR)
BL0265	7.5*	12.1*	9.8*	Fragment of arabinose permease
BL0573	13.1*	11.8*	8.6*	Hypothetical protein
BL1251	11.3*	11.8*	9.1*	Glutamyl-tRNA synthetase
BL1311	5.7*	8.8*	10.2*	Hypothetical protein with LysM domain of membrane-bound lytic murein transglycosylases
BL0571	10.7*	8.7*	5.4*	Putative esterase
BL0174	9.3*	8.1*	7.3*	Narrowly conserved hypothetical protein
BL0572	7.3*	7.7*	5.8*	Hypothetical membrane protein with unknown function
BL0599	5.1*	7.5*	6.9*	Hypothetical protein
BL0662	6.1*	7.5*	7.2*	Hypothetical protein
BL0003	7.9*	7.4*	3.9*	Hypothetical protein
BL1727	4.8*	7.3*	5.9*	Hypothetical protein
BL0523	4.5*	7.1*	4.8*	Possible xylosidase or glucosidase
BL1673	3.9*	6.7*	4.9*	Possible lactaldehyde reductase
BL0175	4.8*	6.4*	7.0*	Probable AraC/XylS-type transcriptional regulator
BL1309	6.0*	6.3*	3.4	Probable cation efflux protein
BL1164	6.1*	6.1*	2.8	Probable solute binding protein of ABC transporter system for sugars
BL1180	4.4*	6.1*	6.0*	SsrA-binding protein (SmpB)
BL0555	6.4*	6.0*	5.5*	Possible DO serine protease
BL1700	2.8	5.6*	4.3*	Polypeptide deformylase
BL0532	3.9*	5.6*	4.4*	Transmembrane transport protein possibly for shikimate
BL0841	3.8	5.4*	4.9*	Hypothetical protein
BL1491	2.6*	5.4*	2.3	Hypothetical protein
BL1804	4.8*	5.3*	2.9	Hypothetical protein
BL1520	3.3*	5.2*	3.9*	Possible NagC/XylR-type transcriptional regulator
BL0107	4.0*	5.1*	6.1*	Probable LacI-type transcriptional regulator
BL0331	4.4*	5.0*	3.4*	Probable PfkB family carbohydrate (sugar) kinase
BL1198	2.7*	5.0*	3.7*	Probable serine/threonine-protein kinase
BL0661	3.3*	4.9*	6.4*	Hypothetical protein
BL0161	3.2*	4.9*	4.5*	Hypothetical protein
BL0368	4.4	4.9*	3.8*	Hypothetical protein
BL0202	2.2	4.9*	4.7*	Hypothetical protein
BL1189	2.5	4.9*	5.3*	Hypothetical protein
BL0422	5.4*	4.9*	4.8*	Narrowly conserved hypothetical protein
BL0423	3.5*	4.8*	5.8*	Sugar permease of ABC transporter system
BL1293	2.5	4.8*	6.2*	Similar to xylulose kinase
BL1437	3.4*	4.8*	5.1*	Hypothetical cytosolic proteins
BL1803	4.0	4.8*	7.6*	Hypothetical protein
BL0261	2.0	4.8*	4.5*	ABC-type sugar transport systems, permease components
BL1559	5.6*	4.7*	4.1*	Cystathionine gamma-lyase
BL0154	3.7*	4.6*	3.2	ATP binding protein of ABC transporter
BL0189	3.1	4.6*	2.5	Sugar permease of ABC transporter system
BL0002	5.8*	4.5*	4.3*	Chaperone protein (GroEL)
BL1216	2.8*	4.5*	3.6*	Probable transcriptional regulator with cyclic nucleotide-binding domain

*Significantly induced genes ($P < 0.0007$).

†Ratio of gene expression between heat-shock treated cells and control cells.

RNAses. A model supporting this hypothesis was recently proposed in *Caulobacter crescentus*, where SmpB was shown to protect tmRNA from RNAse R degradation *in vitro*, and the levels of SmpB protein were shown to correlate with tmRNA stability *in vivo* (Hong *et al.*, 2005).

Induction of genes with unknown function

Many of the genes whose expression was altered by a temperature increase encode proteins without homology to any members of a COG group (Fig. 2). In an effort to identify new general stress response genes, the 141 hypothetical genes significantly induced at 7 min were compared using BLASTP against a nonredundant bacterial protein database (NCBI). Potential gene orthologs were further considered only if the query sequence displayed an amino acid sequence identity greater than 30% over at least half of its length. For the 103 *Bi. longum* NCC2705 sequences fulfilling these criteria, the literature was searched for published stress-related global gene expression studies on the species carrying corresponding gene orthologs. This analysis resulted in the identification of three NCC2705 genes with a hit in another organism, in which the corresponding orthologous gene was previously shown to be induced under similar heat shock conditions: BL0408 protein displayed 52% identity over 87% of its length with the *Ba. subtilis yvgZ*-encoded unknown protein (Helmann *et al.*, 2001); BL0503 displayed 39% identity over 62% of its length with the *Mycobacterium tuberculosis Rv3654c*-encoded hypothetical protein (Stewart *et al.*, 2002); finally BL0850 was 33% identical over 75% of its length with the *Shewanella oneidensis SO3342*-encoded hypothetical protein (Gao *et al.*, 2004). The presence of potential orthologs of these genes induced under similar heat-stress conditions in relatively distant bacterial species suggests that they might play a common role in the response to this stress stimulus.

Conclusions

This study describes the first genome-wide gene expression profile analysis of a *Bifidobacterium* species in response to heat stress, a condition that is highly relevant to the industrial production of probiotic strains. Results of this study show that 46% of the *Bi. longum* genes were differentially expressed. These include common bacterial stress-responses such as the induction of chaperones and the down-regulation of the translation, cell division and chromosome-partitioning machineries. In parallel, several transcriptional regulators were rapidly induced suggesting their involvement in the observed reshuffle of the gene-expression profile. Some less-characterized responses were also identified, such as the induction of the *trans*-translation machinery, which could play a major role for survival of *Bi. longum* cells under high temperature conditions. Overall, these data significantly contribute to the understanding of *Bi. longum* stress-response mechanisms and build the basis for future more in-depth functional studies.

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References

Barreiro C, Gonzalez-Lavado E, Brand S, Tauch A & Martin JF (2005) Heat shock proteome analysis of wild-type *Corynebacterium glutamicum* ATCC 13032 and a spontaneous mutant lacking GroEL1, a dispensable chaperone. *J Bacteriol* **187**: 884–889.

Campoy S, Salvador N, Cortes P, Erill I & Barbe J (2005) Expression of canonical SOS genes is not under LexA repression in *Bdellovibrio bacteriovorus*. *J Bacteriol* **187**: 5367–5375.

Chermesh I & Eliakim R (2006) Probiotics and the gastrointestinal tract: where are we in 2005? *World J Gastroenterol* **12**: 853–857.

De Angelis M & Gobbetti M (2004) Environmental stress responses in *Lactobacillus*: a review. *Proteomics* **4**: 106–122.

Fernandez De Henestrosa AR, Ogi T, Aoyagi S, Chafin D, Hayes JJ, Ohmori H & Woodgate R (2000) Identification of additional genes belonging to the LexA regulon in *Escherichia coli*. *Mol Microbiol* **35**: 1560–1572.

Flahaut S, Hartke A, Giard JC, Benachour A, Boutibonnes P & Auffray Y (1996) Relationship between stress response toward bile salts, acid and heat treatment in *Enterococcus faecalis*. *FEMS Microbiol Lett* **138**: 49–54.

Gao H, Wang Y, Liu X, Yan T, Wu L, Alm E, Arkin A, Thompson DK & Zhou J (2004) Global transcriptome analysis of the heat shock response of *Shewanella oneidensis*. *J Bacteriol* **186**: 7796–7803.

Hatfield GW, Hung SP & Baldi P (2003) Differential analysis of DNA microarray gene expression data. *Mol Microbiol* **47**: 871–877.

Helmann JD, Wu MF, Kobel PA, Gamo FJ, Wilson M, Morshedi MM, Navre M & Paddon C (2001) Global transcriptional response of *Bacillus subtilis* to heat shock. *J Bacteriol* **183**: 7318–7328.

Hong SJ, Tran QA & Keiler KC (2005) Cell cycle-regulated degradation of tmRNA is controlled by RNase R and SmpB. *Mol Microbiol* **57**: 565–575.

Karzai AW & Sauer RT (2001) Protein factors associated with the SsrA.SmpB tagging and ribosome rescue complex. *Proc Natl Acad Sci USA* **98**: 3040–3044.

Klijn A, Mercenier A & Arigoni F (2005) Lessons from the genomes of bifidobacteria. *FEMS Microbiol Rev* **29**: 491–509.

Lönnstedt I & Speed TP (2002) Linear models and empirical Bayes methods for assessing differential expression in microarray experiments. *Statistica Sinica* **12**: 31–46.

Mascher T, Helmann JD & Unden G (2006) Stimulus perception in bacterial signal-transducing histidine kinases. *Microbiol Mol Biol Rev* **70**: 910–938.

Maus JE & Ingham SC (2003) Employment of stressful conditions during culture production to enhance subsequent cold- and acid-tolerance of bifidobacteria. *J Appl Microbiol* **95**: 146–154.

Moore SD & Sauer RT (2005) Ribosome rescue: tmRNA tagging activity and capacity in *Escherichia coli*. *Mol Microbiol* **58**: 456–466.

Muto A, Fujihara A, Ito KI, Matsuno J, Ushida C & Himeno H (2000) Requirement of transfer-messenger RNA for the growth of *Bacillus subtilis* under stresses. *Genes Cells* **5**: 627–635.

Narberhaus F (1999) Negative regulation of bacterial heat shock genes. *Mol Microbiol* **31**: 1–8.

Pallen MJ & Wren BW (1997) The HtrA family of serine proteases. *Mol Microbiol* **26**: 209–221.

Parche S, Beleut M, Rezzonico E, Jacobs D, Arigoni F, Titgemeyer F & Jankovic I (2006) Lactose-over-Glucose preference in *Bifidobacterium longum* NCC2705: glcP, encoding a glucose transporter, is subject to lactose repression. *J Bacteriol* **188**: 1260–1265.

Picard C, Fioramonti J, Francois A, Robinson T, Neant F & Matuchansky C (2005) Review article: bifidobacteria as probiotic agents – physiological effects and clinical benefits. *Aliment Pharmacol Ther* **22**: 495–512.

Price MN, Huang KH, Alm EJ & Arkin AP (2005) A novel method for accurate operon predictions in all sequenced prokaryotes. *Nucleic Acids Res* **33**: 880–892.

Richmond CS, Glasner JD, Mau R, Jin H & Blattner FR (1999) Genome-wide expression profiling in *Escherichia coli* K-12. *Nucleic Acids Res* **27**: 3821–3835.

Saarela M, Rantala M, Hallamaa K, Nohynek L, Virkajarvi I & Matto J (2004) Stationary-phase acid and heat treatments for improvement of the viability of probiotic lactobacilli and bifidobacteria. *J Appl Microbiol* **96**: 1205–1214.

Sanchez B, Champomier-Verges MC, Anglade P, Baraige F, de Los Reyes-Gavilan CG, Margolles A & Zagorec M (2005) Proteomic analysis of global changes in protein expression during bile salt exposure of *Bifidobacterium longum* NCIMB 8809. *J Bacteriol* **187**: 5799–5808.

Savijoki K, Suokko A, Palva A, Valmu L, Kalkkinen N & Varmanen P (2005) Effect of heat-shock and bile salts on protein synthesis of *Bifidobacterium longum* revealed by [35S]methionine labelling and two-dimensional gel electrophoresis. *FEMS Microbiol Lett* **248**: 207–215.

Schell MA, Karmirantzou M, Snel B *et al.* (2002) The genome sequence of *Bifidobacterium longum* reflects its adaptation to the human gastrointestinal tract. *Proc Natl Acad Sci USA* **99**: 14422–14427.

Schmidt G & Zink R (2000) Basic features of the stress response in three species of bifidobacteria: *B. longum*, *B. adolescentis*, and *B. breve*. *Int J Food Microbiol* **55**: 41–45.

Simpson PJ, Stanton C, Fitzgerald GF & Ross RP (2005) Intrinsic tolerance of *Bifidobacterium* species to heat and oxygen and survival following spray drying and storage. *J Appl Microbiol* **99**: 493–501.

Smyth GK (2004) Linear models and empirical Bayes methods for assessing differential expression in microarray experiments. *Statistical Appl Genet Mol Biol* **3**. Article 3.

Stewart GR, Wernisch L, Stabler R, Mangan JA, Hinds J, Laing KG, Young DB & Butcher PD (2002) Dissection of the heat-shock response in *Mycobacterium tuberculosis* using mutants and microarrays. *Microbiology* **148**: 3129–3138.

Stintzi A (2003) Gene expression profile of *Campylobacter jejuni* in response to growth temperature variation. *J Bacteriol* **185**: 2009–2016.

Susin MF, Baldini RL, Gueiros-Filho F & Gomes SL (2006) GroES/EL and DnaK/J have distinct roles in stress responses and during cell cycle progression in *Caulobacter crescentus*. *J Bacteriol* **188**: 8044–8053.

Szajewska H, Setty M, Mrukowicz J & Guandalini S (2006) Probiotics in gastrointestinal diseases in children: hard and not-so-hard evidence of efficacy. *J Pediatr Gastroenterol Nutr* **42**: 454–475.

Tatusov RL, Koonin EV & Lipman DJ (1997) A genomic perspective on protein families. *Science* **278**: 631–637.

Ventura M, Canchaya C, Zink R, Fitzgerald GF & van Sinderen D (2004a) Characterization of the *groEL* and *groES* Loci in *Bifidobacterium breve* UCC 2003: genetic, transcriptional, and phylogenetic analyses. *Appl Environ Microbiol* **70**: 6197–6209.

Ventura M, van Sinderen D, Fitzgerald GF & Zink R (2004b) Insights into the taxonomy, genetics and physiology of bifidobacteria. *Antonie Van Leeuwenhoek* **86**: 205–223.

Ventura M, Canchaya C, Bernini V, Del Casale A, Dellaglio F, Neviani E, Fitzgerald GF & van Sinderen D (2005a) Genetic characterization of the *Bifidobacterium breve* UCC 2003 *hrcA* locus. *Appl Environ Microbiol* **71**: 8998–9007.

Ventura M, Fitzgerald GF & van Sinderen D (2005b) Genetic and transcriptional organization of the *clpC* locus in *Bifidobacterium breve* UCC 2003. *Appl Environ Microbiol* **71**: 6282–6291.

Ventura M, Kenny JG, Zhang Z, Fitzgerald GF & van Sinderen D (2005c) The *clpB* gene of *Bifidobacterium breve* UCC 2003: transcriptional analysis and first insights into stress induction. *Microbiology* **151**: 2861–2872.

Ventura M, Zhang Z, Cronin M, Canchaya C, Kenny JG, Fitzgerald GF & van Sinderen D (2005d) The ClgR protein regulates transcription of the *clpP* operon in *Bifidobacterium breve* UCC 2003. *J Bacteriol* **187**: 8411–8426.

Ventura M, Zink R, Fitzgerald GF & van Sinderen D (2005e) Gene structure and transcriptional organization of the *dnaK* operon of *Bifidobacterium breve* UCC 2003 and application of the

operon in bifidobacterial tracing. *Appl Environ Microbiol* **71**: 487–500.

Weiner J III, Zimmerman CU, Gohlmann HW & Herrmann R (2003) Transcription profiles of the bacterium *Mycoplasma pneumoniae* grown at different temperatures. *Nucleic Acids Res* **31**: 6306–6320.

Withey JH & Friedman DI (2003) A salvage pathway for protein structures: tmRNA and trans-translation. *Annu Rev Microbiol* **57**: 101–123.

Yuan J, Zhu L, Liu X *et al.* (2006) A proteome reference map and proteomics analysis of *Bifidobacterium longum* NCC2705. *Mol Cell Proteomics* **5**: 1105–1118.

Yura T, Kanemori M & Morita MT (2000) The heat shock response: regulation and function. *Bacteria Stress Responses* (Storz GT & Hengge-Aronis R, eds.), pp. 3–17. ASM Press, Washington, DC.

Supplementary material

The following supplementary material is available for this article:

Table S1. Oligonucleotide primers used for qRT-PCR.

Table S2. *Bifidobacterium longum* NCC2705 gene expression values at 3, 7 and 12 minutes after beginning of the HS.

Table S3. 50 genes displaying to the strongest level of repression after 7 minutes at 50 °C.

Table S4. List of genes belonging to the COG functional category K induced after 7 minutes at 50 °C.

Fig. S1. Comparison of the survival rate between control and heat-treated cultures during the analyzed time period, measured by plate counting method. Values for each group at each time point represent the average of three experimental replicates.

Fig. S2. Validation of microarray results by real-time qRT-PCR. Sports represent single $\log_2$-transformed relative gene expression ratios between heat-stressed and control bacteria, measured by microarray (X-axis) or by real-time RT-PCR (Y-axis) at 3 (○) 7 (✻) and 12 minutes (♦) after beginning of the heat shock.

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